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High-Gravity Brewing: Effects of Nutrition on Yeast Composition, Fermentative Ability, and Alcohol Production

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A number of economic and product quality advantages exist in brewing when high-gravity worts of 16 to 18% dissolved solids are fermented. Above this level, production problems such as slow or stuck fermentations and poor yeast viability occur. Ethanol toxicity has been cited as the main cause, as brewers' yeasts are reported to tolerate only 7 to 9% (vol/vol) ethanol. The inhibitory effect of high osmotic pressure has also been implicated. In this report, it is demonstrated that the factor limiting the production of high levels of ethanol by brewing yeasts is actually a nutritional deficiency. When a nitrogen source, ergosterol, and oleic acid are added to worts up to 31% dissolved solids, it is possible to produce beers up to 16.2% (vol/vol) ethanol. Yeast viability remains high, and the yeasts can be repitched at least five times. Supplementation does not increase the fermentative tolerance of the yeasts to ethanol but increases the length and level of new yeast cell mass synthesis over that seen in unsupplemented wort (and therefore the period of more rapid wort attenuation). Glycogen, protein, and sterol levels in yeasts were examined, as was the importance of pitching rate, temperature, and degree of anaerobiosis. The ethanol tolerance of brewers' yeast is suggested to be no different than that of sake or distillers' yeast.

In traditional brewing, worts of 11 to 12% dissolved solids are fermented to produce beers of 4 to 5% (vol/vol) ethanol. Recently, high-gravity brewing at a limit of 16 to 18% dissolved solids has become popular due to advantages such as increased plant efficiency; reduced energy, labor, and capital costs; use of higher adjunct ratios; improved smoothness, flavor, and haze stability of beer; and increased ethanol yields per unit of fermentable extract (15, 38).

Attempts to ferment worts above 18% dissolved solids have proven to be difficult, largely because of problems with yeast viability and slow and incomplete fermentations (9, 37). Both ethanol toxicity (9, 37) and high osmotic pressure levels (29) have been implicated as the limiting factors.

In recent studies with normal-gravity worts, it has been indicated that the factors described above could be related to nutritionally induced growth problems. In sharp contrast to the long-held belief in brewing that the bulk of wort attenuation is done by nongrowing cells (22), it is now clear that the specific rate of sugar utilization by growing yeast cells in fermentation is substantially higher than that of nongrowing cells (22). Thus, when the period of new cell mass production ceases during fermentation, the rate of attenuation also slows dramatically (by as much as 33-fold) (22). It therefore follows that in high-gravity brewing, both the length and level of new cell synthesis must be increased over the amounts found in normal-gravity brewing to have rapid fermentation. It is our belief that the concentration of the nutrients most likely to limit growth (e.g., oxygen and assimilable nitrogen) must certainly be increased in such worts.

Such nutritional concerns have not been considered in brewing practice. The most widely used method to prepare high-gravity worts has been the addition of corn syrups to the kettle (15). Such syrups are virtually devoid of any nitrogenous nutrients, and their use effectively decreases the proportion of all noncarbohydrate nutrients in the wort (20). Although worts made only of malt contain excess assimilable nitrogen (22), literature reports with normal-gravity worts have illustrated that nitrogen-induced problems, mimicking those found in high-gravity brewing, are found when more and more of the extract is substituted with adjunct (19, 22).

Oxygen is required by brewing yeasts for the synthesis of sterols and unsaturated fatty acids (1, 2). Such lipids are present in suboptimal concentrations even in normal-gravity worts (7). In high-gravity worts, oxygen availability is diminished even further due to the decreasing solubility of oxygen with increasing wort gravity (3). As reproductive growth ceases once a limiting value of sterols is reached in yeasts (22), this lowered O_2 solubility in high-gravity worts only increases the probability of growth-related attenuation problems.

Recently, our research has demonstrated that a combination of increased pitching rates (5) and nutritional supplementation (6) can permit the rapid fermentation of worts up to 28% dissolved solids. Specifically, supplementation of these worts with yeast extract (as a source of assimilable nitrogen)-ergosterol-oleic acid (to provide a sterol and unsaturated fatty acid) at a pitching rate of 20×10^6 to 30×10^6 CFU per ml resulted in the production of up to 15% (vol/vol) ethanol at 14°C within 5 days (compared with 2 weeks in unsupplemented worts). The improvements were a result of prolonged and increased production of yeast cell mass arising from supplementation (6). This demonstrates that brewers' yeasts, without any genetic manipulation or strain improvement, are tolerant to at least 15% (vol/vol) ethanol.

The results presented here describe continued research on the importance of nutritional supplementation in high-gravity fermentations.

MATERIALS AND METHODS

Brewing yeast. Fresh slurries of a commercial lager yeast, *Saccharomyces uvarum* (*carlsbergensis*), were collected just before use.

High-gravity worts. The 11.5°P (22.5% corn grit adjunct) commercial wort used was autoclaved to remove protein and to allow yeast cell mass assessment without interference

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from precipitated protein (trub). Fermentations are unaffected by this step, as brewers' yeasts cannot use protein as a nitrogen source (19, 31). For the preparation of worts of higher original gravity, Casco syrup no. 1636U was added (Canada Starch Co. Ltd.). The manufacturer's carbohydrate specifications for this syrup are 19.4% DP4+, 10.7% DP3, 39.0% DP2, and 30.9% DP1. The syrup is virtually nitrogen free, containing only 0.03 to 0.06% (wt/vol) protein. In special instances, maltose or glucose was used in place of the syrup.

Chemicals. Ergosterol (Sigma Chemical Co., St. Louis, Mo.) was dissolved in a mixture of ethanol-Tween 80 (1). Tween 80 (Sigma) serves as a source of unsaturated fatty acid, containing 0.6% free oleic acid (8). Yeast extract (Difco Laboratories, Detroit, Mich.) was used as the nitrogen supplement.

Fermentation conditions. All fermentations were in 1- and 2-liter jacketed Wheaton Celstir fermentors and stirred at 90 rpm by a Wheaton model III Biostir 6. The temperature was maintained at 14°C with a Haake model D-3G circulator. Unless otherwise stated, anaerobiosis, as experienced industrially by CO_2 purging, was maintained by continually flushing the headspace with nitrogen gas at a rate of 30 ml/min. In special instances, however, semianaerobic fermentations were carried out where there was no nitrogen flushing, and foam plugs were used in the sampling ports. Pitching rates of 20×10^6 to 30×10^6 CFU per ml were normally used.

Viable counts. Viable counts were determined by the membrane filtration technique (5).

Total solids and dry weight determinations. Carbohydrate utilization was approximated by removing yeasts by centrifugation and assessing total solids in the supernatant (5).

For dry weight determinations during manometric analyses, cell pellets were washed three times in M/15 KH_2PO_4 buffer (pH 4.5) and then resuspended in the same solution. These suspensions were used for manometry, and triplicate 3-ml samples of the suspensions and of the resuspended KH_2PO_4 were transferred to preweighed aluminum foil pans. Pans were dried to a constant weight at 105°C (corrected for buffer weight), and the cell dry weight (in milligrams per milliliter) was calculated. For dry weight determinations of the fermentation samples, distilled water was used in the place of M/15 KH_2PO_4 buffer.

Ethanol assays. Ethanol was measured enzymatically with alcohol dehydrogenase, as outlined in Sigma Technical Bulletin number 331 U.V.

FAN. Free alpha amino nitrogen (FAN) levels in wort and beer were determined by the European Brewing Convention ninhydrin method (11) at 570 nm. Glycine (Fisher Scientific Co., Pittsburgh, Pa.) was used to prepare a standard curve, and the results were calculated by linear regression.

Manometric analyses. The fermentative power of washed yeast samples was determined in a Warburg respirometer by the European Brewing Convention Analytica Microbiologica manometric technique (12). The single alteration to the previously published method was the continual maintenance of anaerobic conditions (via nitrogen flushing) in the samples from the time of sampling onward. Fermentative power ($Q^{N_2}_{CO_2}$) was expressed as the number of microliters of CO_2 given off under a nitrogen atmosphere per hour per milligram (dry weight) of yeast sample; this was measured over a 90-min period. To determine fermentative tolerance to ethanol, ($Q^{N_2}_{CO_2}$) values were determined in the presence of 0, 5, 10, 15, and 20% (vol/vol) ethanol. From these, the concentration of ethanol resulting in a 50% reduction of the ($Q^{N_2}_{CO_2}$) value found in the absence of ethanol (hereafter referred to as the IF_{50}) was then calculated.

Cellular analyses. Protein was estimated spectrophotometrically at 750 nm (23), with bovine serum albumin (Sigma) used as the standard protein.

Glycogen levels were determined spectrophotometrically at 660 nm by an idoine-potassium iodide staining method (33). Rabbit liver glycogen (Sigma) was used as the standard.

Total cell sterol levels were measured by the modified Liebermann-Buchard reaction (13). Ergosterol (Sigma) was used as the standard.

High-pressure liquid chromatographic analyses. A Beckman model 420 high-pressure liquid chromatographic system was used. Detection was carried out with an Altex 156 refractive index detector, and integration was carried out with a Hewlett Packard 3390A integrator. The heating block (model CH-20; Scientific Systems Inc.) contained a μ-Spherogel column (Beckman Instruments, Inc.) designed for carbohydrate analysis. Column dimensions were inner diameter, 7.5 mm; outer diameter, 10 mm; and length, 300 mm. The packing was 7.5% cross-linked calcium-loaded sulfonated polystyrene divinylbenzene resin. The operative temperature was 80°C, with degassed, distilled, and filtered (pore size, 0.45 μm) water used as the solvent. The flow rate was 0.6 ml/min at 400 lb/in^2. Sorbitol was used as the internal standard at a constant concentration of 3.0 mg/ml. Sucrose had a retention time identical to that of maltose, but as it only constitutes 1.9% of the DP2 in these worts (31), it was not considered separately from the maltose. The ratios of peak height responses of each individual sugar to the sorbitol internal standard were plotted against the concentrations. The concentrations of the sugar in the wort and beer samples were obtained by calculating their ratios of peak heights

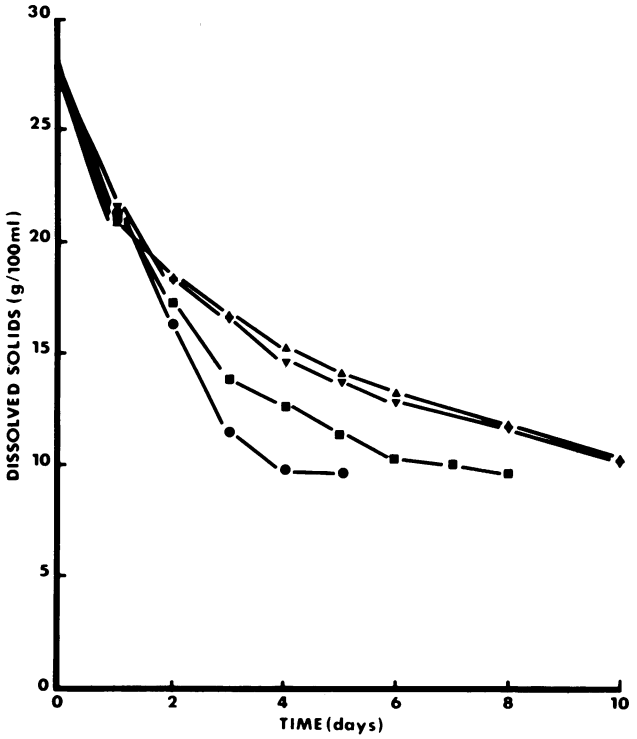

FIG. 1. Dissolved solids versus time during the semianaerobic fermentation of high-gravity worts that were unsupplemented (▲), 1% yeast extract supplemented (■), 40 ppm ergosterol and 0.4% Tween 80 supplemented (▼), or 1% yeast extract with 40 ppm ergosterol and 0.4% Tween 80 supplemented (●).

relative to that of the sorbitol internal standard and then by referring to the calibration plot and multiplying by the dilution factor.

RESULTS

Evidence for the stimulatory role of oxygen under semianaerobic conditions and for the importance of nutritional supplementation is presented in Fig. 1. Under semianaerobic conditions, the added yeast extract was stimulatory (it was not stimulatory anaerobically [6]), as O_2 was available for lipid synthesis under semianaerobic conditions. Anaerobically, lipid addition (40 ppm ergosterol and 0.4% Tween 80) enhanced fermentation (6), whereas under the conditions used in this study, the same added lipids were not stimulatory (as oxygen was being introduced into the unsupplemented wort), and the wort attenuated at the same protracted rate as under unsupplemented conditions. With complete supplementation, however, the fermentation time of this 27% dissolved solid wort was reduced to 4 days, and a synergistic effect of the two supplements was seen as described previously (6).

Analysis of the levels of FAN before and after fermentation illustrates that in the presence of added lipids, useable nitrogen is, indeed, growth limiting and that the simultaneous presence of preformed lipids or oxygen is required for full utilization of such nitrogenous constituents. Anaerobically, only 106 and 107 mg of FAN per liter were utilized in the unsupplemented and 1% yeast extract-supplemented worts, respectively (despite the ca. 400-mg/liter increase in FAN levels arising from yeast extract addition). In the lipid-supplemented wort, 165 mg of the original 213 mg of the available FAN per liter was utilized. However, when both of the supplements were present, 289 mg of FAN per liter was utilized (36% more FAN than the starting total in unsupplemented wort). Increased cell growth (data not shown) and a significantly reduced fermentation time resulted.

Semianaerobically, however, when there is contact with air, more of the available FAN was utilized in the unsupplemented wort (128 versus 106 mg/liter), as well as in the yeast extract-supplemented wort (229 versus 107 mg/liter) than anaerobically, which explains the stimulation seen in the rate of fermentation. Maximum utilization of FAN (286 mg/liter) was only seen when both the yeast extract and the lipids were present (73% more FAN than the starting total in unsupplemented wort).

High-pressure liquid chromatographic analysis of the worts and beers from the fermentations described above (data not shown) demonstrated that attenuation of the fermentable carbohydrates (i.e., glucose, fructose, maltose, and maltotriose) is complete in fully supplemented worts. Low levels of maltose and maltotriose (0.85 and 0.99 g per 100 ml) remained in this beer. These levels are normal after a primary fermentation and would be reduced further during subsequent aging (31). In the unsupplemented fermentation, however, considerable amounts of fermentable carbohydrate, especially maltose (2.65 g per 100 ml), still remained even after 8 days.

The cellular levels of glycogen and sterols in yeasts obtained from the end of 27% dissolved solids wort fermentations were found to be significantly influenced by the use of anaerobic or semianaerobic conditions (Table 1). Although protein levels were similar in both sets of fermentations, the levels of cellular glycogen were significantly lower and the levels of cellular sterols were higher in each of the semianaerobic fermentations.

In the anaerobic fermentations, the yeast crop from the fully supplemented wort had the greatest level of cellular sterols (0.56%) but the lowest level of glycogen (10.5%). When followed throughout the course of fermentation, supplementation resulted in higher maximum levels of sterols (0.85 versus 0.63%), and as growth continued, the sterol concentration was diluted. The final levels reached were higher in the supplemented yeasts (0.61%) than in the unsupplemented yeasts (0.48%). In the case of glycogen, however, peak levels were higher in yeasts from the unsupplemented fermentation (21.0 versus 17.4%) and remained at higher levels by the end of the fermentation (19.8 versus 12.5%).

The possibility remained that nutritional supplementation was also enhancing the rate and extent of fermentation by increasing the tolerance of yeasts to ethanol. Unsaturated fatty acids, in particular, have been demonstrated to play a role in the alcohol tolerance of yeasts (35). Table 2 records the ($Q_{CO_2}^{N_2}$) values of yeasts sampled throughout the fermentation of unsupplemented and fully supplemented worts, and Table 3 provides yeast cell mass and other fermentation data. In virtually every instance, regardless of the concentration of ethanol present during the assay, the fermentative power of unsupplemented yeasts was equal to or greater than that of the yeasts in the supplemented fermentations. Average IF_{50} values for the unsupplemented yeasts were 11.8% (vol/vol) ethanol (±1.20%), as compared with 11.4% (vol/vol) for the supplemented yeasts (±0.5%).

Previously, 14.2% (vol/vol) ethanol was produced in fully supplemented wort when maltose, which can be entirely fermented, was used as the brewing adjunct instead of corn

TABLE 1. Percent glycogen, protein, and total sterols (on a dry weight basis) in the crops of yeasts from high-gravity fermentations with or without nutritional supplementation under anaerobic or semianaerobic conditions

Treatment	% of the following after anaerobic fermentations:			% of the following after semianaerobic fermentations:		
	Glycogen	Protein	Sterols	Glycogen	Protein	Sterols
Unsupplemented wort	28.4	42.1	0.51	6.7	45.0	0.85
1% yeast extract supplemented	25.8	45.6	0.27	7.0	47.9	1.01
40 ppm ergosterol–0.4% Tween 80 (vol/vol) supplemented	26.5	36.9	0.40	9.5	43.1	1.09
1% yeast extract–40 ppm ergosterol–0.4% Tween 80 (vol/vol) supplemented	10.5	43.5	0.56	7.8	44.6	0.89

TABLE 2. $Q_{CO_2}^{N_2}$ values of washed yeasts removed from the anaerobic fermentation of unsupplemented and supplemented 27% dissolved solids wort[a]

Day	$Q_{CO_2}^{N_2}$ values[b] in the presence of the following % (vol/vol) ethanol									
	Unsupplemented					Supplemented				
	0	5	10	15	20	0	5	10	15	20
0	202	140	108	34	27	194	155	97	35	23
1	303	267	173	93	41	311	244	161	98	64
2	182	140	94	47	33	182	145	105	44	39
3	183	173	117	64	44	181	140	100	55	44
4	171	162	110	65	31	167	126	84	55	25
5	163	136	92	48	18	171	125	82	52	34
7	130	104	72	42	29	135	104	76	40	31
9	108	100	72	41	28	ND[c]	ND	ND	ND	ND
13	101	70	44	25	14	ND	ND	ND	ND	ND

[a] Supplemented worts consisted of 0.8% yeast extract–24 ppm ergosterol–0.24% (vol/vol) Tween 80. Reduced levels of supplements had no negative effects on the fermentation (6).
[b] $Q_{CO_2}^{N_2}$ values are in microliters of CO_2 per hour per milligram of cells (dry weight).
[c] ND, Not determined.

syrup, which is only 70 to 80% fermentable (6). To determine the original gravity limit to maltose adjunct worts, worts with up to 35% dissolved solids were fermented (Fig. 2). Although yeast viability remained high in all worts (data not shown), only those with up to 31.9% dissolved solids reached end gravity, producing 16.2% (vol/vol) ethanol.

It is important in brewing that a portion of the yeast crop from the end of one fermentation is able to be used to initiate a fresh fermentation. Supplemented 28% maltose adjunct worts were repitched five times without any decrease in fermentative ability, with the fermentations being complete within 5 days in all cases (data not shown). Yeast viability and cell mass production remained high throughout the five runs, and high-pressure liquid chromatographic analysis of the beers after runs 1 and 5 indicated normal attenuation of all the fermentable carbohydrates (<0.2% maltose, <1.4% maltotriose). Final ethanol concentrations of 14.2, 13.6, 14.3, 13.7, and 14.2% (vol/vol) were reached in runs 1 through 5, respectively. Analysis of glycogen and sterols in the yeasts from the resulting crops in these fermentations indicated that as in the case of single-batch fermentations (Table 1), the levels of glycogen were ca. 50% lower in the yeasts from the supplemented fermentations, whereas the levels of the sterols were at least 15% higher.

TABLE 3. Fermentation parameters during the anaerobic fermentation of the unsupplemented and supplemented 27% dissolved solids worts used to determine yeast $Q_{CO_2}^{N_2}$ and IF_{50} values[a]

Day	Dissolved solids (g/100 ml)		Yeast dry wt (mg/ml)		Yeast viability (CFU/ml × 10^{-6})	
	Unsupplemented	Supplemented	Unsupplemented	Supplemented	Unsupplemented	Supplemented
0	27.2	27.7	3.4	3.4	21.4	22.4
1	24.0	23.9	6.9	7.5	34.6	35.2
2	19.0	17.5	9.2	11.4	44.6	54.7
3	17.9	12.6	8.6	13.8	42.1	68.2
4	16.8	10.3	9.0	15.0	45.3	68.0
5	16.6	9.7	9.9	15.9	50.3	73.9
7	15.5	9.6	9.3	14.8	51.7	73.9
9	14.4	ND[b]	9.4	ND	44.7	ND
13	11.8[c]	ND	8.9	ND	48.6	ND

[a] Supplemented worts consisted of 0.8% yeast extract–24 ppm ergosterol–0.24% (vol/vol) Tween 80.
[b] ND, Not determined.
[c] The fermentation eventually stuck at 11.1 g per 100 ml of dissolved solids.

The influence of temperature on these high-gravity wort fermentations was assessed in light of recent reports on the relationship between it and alcohol tolerance. Supplemented high-gravity corn syrup adjunct worts fermented at 14, 20, 25, and 30°C proceeded to a faster completion as the temperature increased (Fig. 3). However, when yeast viability was followed to day 5, it was extremely low in the fermentations at higher temperatures (inset, Fig. 3), despite the production of similar levels of ethanol in each (ca. 11.4% [vol/vol]). At 14°C, yeast viability remained relatively unchanged, even 10 to 14 days after the completion of fermen-

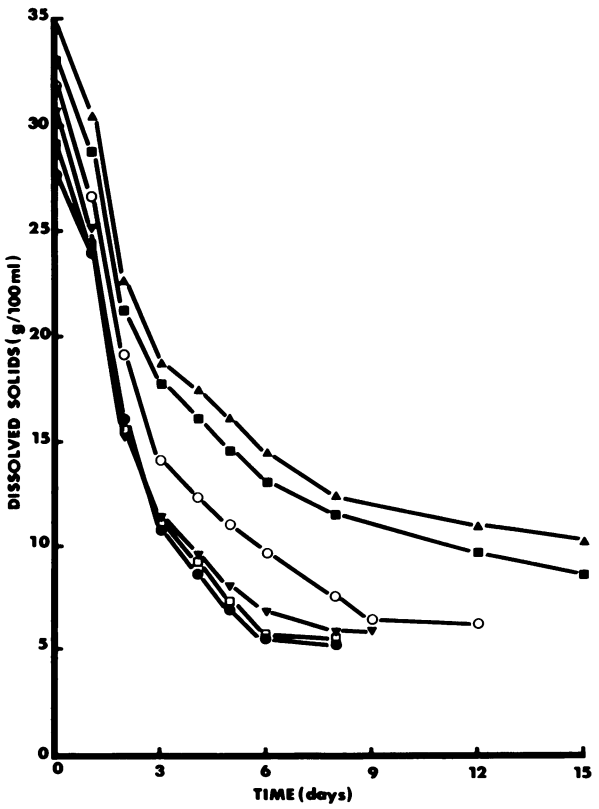

FIG. 2. Dissolved solids versus time during the anaerobic fermentation of fully supplemented 27.9% (●), 29.2% (□), 30.9% (▼), 31.9% (○), 33.3% (■), and 35% (▲) dissolved solids worts.

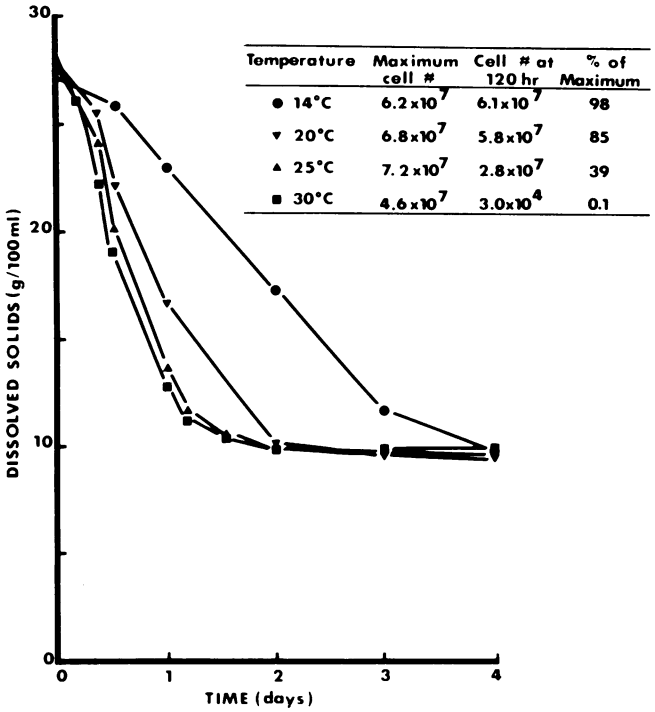

FIG. 3. Dissolved solids versus time during the anaerobic fermentation of 0.8% yeast extract with 24 ppm ergosterol–0.24% (vol/vol) Tween 80-supplemented worts at 14, 20, 25, and 30°C.

tation (data not shown). The poor yeast viability seen at the higher temperatures would certainly preclude its use for repitching in brewing.

The importance of pitching rate in the fermentation of high-gravity corn syrup adjunct worts was determined. It has been previously reported that yeast viability and fermentative ability at 0 to 12 h can be improved in unsupplemented worts by using higher pitching rates (5). When supplemented 29% dissolved solid worts were examined (Table 4), the fermentations proceeded to completion faster as the pitching rate increased up to the inoculum of 2.2×10^7 CFU/ml (the fermentation pitched with an inoculum of 3.1 to 10^7 CFU/ml fermented at virtually the same rate). All five pitching rates, however, resulted in fermentation times of 1 week or less.

In unsupplemented high-gravity worts, however (Table 4), the pitching rate was of more significance. Only at the two highest rates did the fermentations proceed to completion (although 17 to 18 days were required), whereas at the four lower pitching rates, the fermentations became stuck or were only very slowly fermenting (sluggish), even after 3 weeks. Pitching rate, then, can also influence the rate and extent of attenuation, especially in unsupplemented worts.

TABLE 4. Influence of pitching rate on the fermentation of unsupplemented and supplemented 29% dissolved solids high-gravity wort

Pitching rate and days to end fermentation in the following worts[a]:			
Unsupplemented		Supplemented	
Pitching rate (CFU/ml)	Time (days)	Pitching rate (CFU/ml)	Time (days)
3.2×10^6	Sluggish at 21 (14%)[b]	4.6×10^6	7
6.8×10^6	Sluggish at 21 (12.5%)	9.0×10^6	6
1.4×10^7	Stuck at 18 (11.5%)	1.6×10^7	5
2.6×10^7	Stuck at 18 (11.5%)	2.2×10^7	5
3.7×10^7	17–18	3.1×10^7	5
4.4×10^7	17–18		

[a] End gravity was 10% dissolved solids.
[b] Percent dissolved solids at the day indicated.

Alternative sources to be used in place of the addition of preformed lipids and yeast extract were explored. It is indeed possible to entirely replace the need for preformed lipids by sparging with oxygen during the first 2 days of fermentation (4 h per day with 100 ml of oxygen gas per min) (Fig. 4). Cell mass production increased 18% (3.2 mg/ml) in the oxygenated fermentation over that seen in the fermentation provided with preformed lipids, resulting in a somewhat lower final concentration of ethanol (11.5 versus 12.4% [vol/vol]). Final sterol concentrations were 1.14 and 0.71% in the yeasts provided with oxygen and preformed lipids, respectively. The timing of the application of the oxygen was found to be significant, as oxygenation during days 2 through 4 of fermentation was not as effective as during days 0 through 2.

The requirement for yeast extract could be reduced by using more malt. For example, instead of using the 11.5% (22.5% corn adjunct) normal-gravity wort as a base, an all-malt, normal-gravity wort was used. Corn syrup was used to adjust the wort to 31%. It was then possible to decrease the level of yeast extract from 0.8 to 0.2% without decreasing the rate or extent of fermentation (data not shown). The additional FAN contained in malt (Table 5) provided sufficient levels of FAN even in the 0.2% yeast-supplemented fermentation. Final ethanol concentrations of 9.0, 12.2, 12.4, and 12.9% (vol/vol) were reached in the unsupplemented,

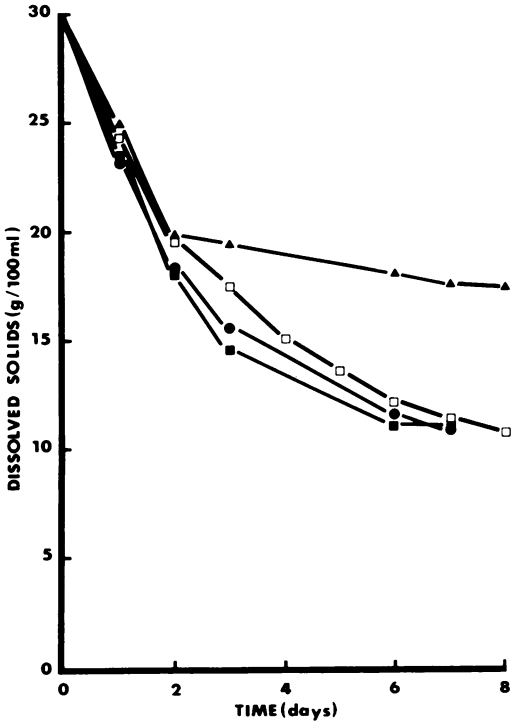

FIG. 4. Dissolved solids versus during the anaerobic fermentation of unsupplemented 30% wort (▲), 0.8% yeast extract supplemented wort treated with 24 ppm ergosterol–0.24% (vol/vol) Tween 80 (●), 4-h periods of oxygenation during days 0, 1, and 2 (■), or 4-h periods of oxygenation during days 2, 3, and 4 (□).

TABLE 5. FAN levels during the anaerobic fermentation of 31% corn syrup adjunct worts (derived from an all-malt wort base) with or without various nutritional supplements

Day	FAN levels (mg/liter) with the following wort treatments:			
	Unsupplemented	24 ppm ergosterol–0.24% (vol/vol) Tween 80	0.8% yeast extract–24 ppm ergosterol–0.24% vol/vol) Tween 80	0.2% yeast extract–24 ppm ergosterol–0.24% (vol/vol) Tween 80
0	250	245	526	311
1	181	144	464	201
2	153	113	384	144
3	148	96	358	118
5	146	96	367	113
8	153	98	376	122
12	170	100	376	122

lipids only supplemented, 0.8% yeast extract plus lipids supplemented, and 0.2% yeast extract plus lipids supplemented worts, respectively.

When the lipids and yeast extract were simultaneously replaced or reduced by oxygenation and increased malt levels, the fermentation of high-gravity maltose adjunct 29% dissolved solids worts proceeded at the same rapid rate (Fig. 5).

DISCUSSION

Regular production strains of brewers' yeasts can, with appropriate nutritional supplementation, produce up to 16.2% (vol/vol) ethanol in the batch fermentation of worts of

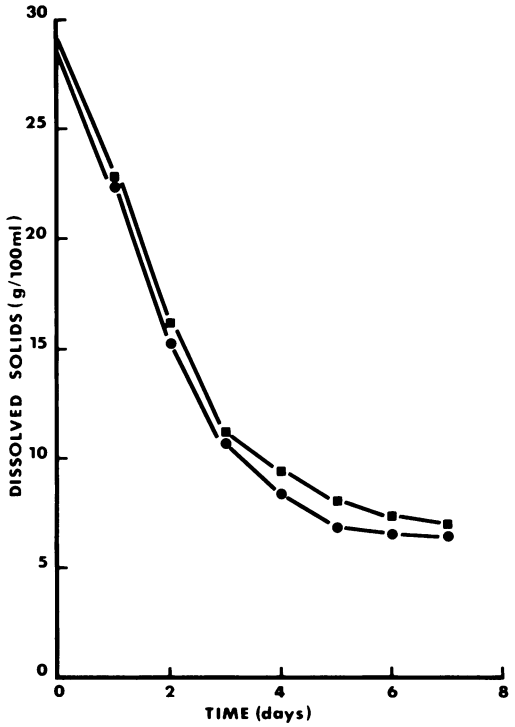

FIG. 5. Dissolved solids versus time during the anaerobic fermentation of maltose adjunct worts. The worts were either prepared for an all-malt base and supplemented with 0.2% yeast extract and oxygenated during days 0, 1, and 2 (100 ml of O_2 gas per min) (●) or prepared from the 22.5% adjunct base and supplemented with 0.8% yeast extract with 24 ppm ergosterol–0.24% (vol/vol) Tween 80 (■).

31% dissolved solids. This occurs at 14°C without incremental feeding, with high yeast crop viability, and within normal brewing time periods (i.e., less than 1 week). The generalization, then, that strains of *Saccharomyces* used in brewing have only moderate alcohol tolerance (9) compared with strains used in distilleries (16) does not hold true from a production viewpoint. Ethanol concentrations of nearly 16.0% (vol/vol) are considered high, even in the distilling industry (16).

For brewing yeasts to produce these high levels of ethanol without an excessively long or stuck fermentation, the wort must contain two types of nutritional supplements: a nitrogen source (provided in this study by yeast extract) and a combination of sterol (provided in this study by ergosterol, the most prevalent sterol in *Saccharomyces* spp. [34]) with unsaturated fatty acid (provided in this study by the oleic acid fraction of Tween 80). It is important that both supplements be provided simultaneously; the fermentation of high-gravity worts then proceeds rapidly under both anaerobic (6) and semianaerobic conditions (Fig. 1).

These supplements act by increasing the amount of new yeast cell mass synthesis over the levels seen in unsupplemented fermentations (Table 3). During wort fermentations, the rate of sugar conversion into ethanol is considerably higher in growing yeast cells (up to 2.0 g/h per g of yeasts) than it is in stationary-phase yeast cells (as low as 0.06 g/h per g of yeasts) (21, 22). Clearly, it is not realistic to expect the rapid and complete attenuation of high-gravity worts by adding only brewing wort syrups to normal-gravity wort bases, as is the current practice (15). By doing so, lipid and nitrogen deficiencies will limit the rate and final level of ethanol production to levels much below the maximum possible, as discussed below.

Lipids that are present in normal-gravity worts in growth-limiting concentrations (7) must be synthesized by the yeasts during the first few hours of fermentation while oxygen is still present (22). However, the decreasing solubility of oxygen in worts with increasing original gravity (3) results in high-gravity worts which, if left unsupplemented, will certainly have oxygen as a growth-limiting nutrient. As demonstrated in this report, in worts with up to 31% dissolved solids, oxygen deficiencies can be overcome by either the addition of 24 ppm ergosterol with 0.24% (vol/vol) Tween 80 as a source of oleic acid or by periods of oxygenation during the fermentation (Fig. 4 and 5). On an industrial scale, the latter would likely be a more economical option.

In low-gravity worts (10 to 12% dissolved solids), a minimum level of 150 mg of FAN per liter is required to permit rapid and complete attenuation (24). Virtually all of this nitrogen is utilized within the first 24 h of fermentation (31), at which point active yeast growth stops (19). In the unsupplemented high-gravity worts used in this study, levels of FAN ranged from 165 to 250 mg/liter. This was not nearly enough to support the necessary degree of cell growth required to rapidly and completely ferment worts as much as three times more concentrated than normal-gravity worts. When yeast extract was incorporated into fully supplemented worts, considerably more FAN was utilized by the yeasts than was even available in total in the unsupplemented worts (as much as 320 mg of FAN per liter). The high levels of yeast extract required can be drastically reduced by the use of an all-malt base (Fig. 5).

It is apparent, therefore, that a frequently mentioned advantage of high-gravity brewing, namely, the increased yield of ethanol resulting from the nonproportional increase in cell mass production with the increase in wort gravity (30),

is in fact an important disadvantage in worts above the current commercial limit of 16 to 18% dissolved solids. Although the use of higher levels of non-nitrogenous and cheaper adjuncts is possible in worts up to this limit, above these levels, nutrient deficiencies will prematurely terminate growth and therefore lead to significantly prolonged and in some cases even stuck fermentations. In the past, such problems have been attributed to the toxic influence of ethanol (9, 37) when in fact growth-limiting levels of nitrogen and oxygen have not permitted sufficient new yeast cell mass synthesis. Indeed, it appears that *Saccharomyces carlsbergensis* can be repitched repeatedly in 30% dissolved solids wort, producing up to 14.2% (vol/vol) ethanol each time, without any increase in the time required for fermentation or decrease in the extent of attenuation. In unsupplemented worts as low as 15.3% dissolved solids (with 40% adjunct), deteriorations in the rate of attenuation, the end gravity reached, and the final ethanol concentration obtained have been observed, even though the first generation gave normal and complete attenuation (18). The claim that ethanol concentrations as low as 8 to 9% (vol/vol) are sufficiently high to kill off a large enough portion of the yeast crop to prevent its use for repitching (35) is therefore not accurate.

Supplementation was shown to have no influence on the fermentative tolerance of the yeasts to ethanol (Table 2). This possibility was explored because sake yeasts only produce the 20 to 23% (vol/vol) levels of ethanol if the proteolipid component of *Aspergillus oryzae* is present (17). In other strains of *Saccharomyces*, exposure to 0.5 to 1.5 M ethanol causes an increase in the proportion of monounsaturated fatty acyl residues in cellular phospholipids (4). Also enhanced are tolerances to the effects of ethanol on generation time, viability, and solute uptake when enrichment with a $C_{18:2}$ unsaturated fatty acid rather than a $C_{18:1}$ unsaturated fatty acid was carried out (35). The early rise and then continued decline in fermentative power values has been attributed to an increase in the cellular levels of hexokinase early in the fermentation, followed by gradual irreversible inhibition and denaturation of hexokinase activity by the buildup of intracellular ethanol levels with time (27).

As the ethanol concentration in this Warburg assay increased up to 20% (vol/vol), so did the degree of inhibition of glycolytic activity. The relationship between the activity remaining (relative to the 0% [vol/vol] control) and the concentration of ethanol was found to be linear. However, when the IF_{50} values of these plots were calculated, little difference between the two sets of yeasts was seen at any point throughout the fermentation. The values fluctuated within the range of 10.1 to 13.6% (vol/vol) ethanol, with the highest IF_{50} values in fact being observed in the yeasts from the unsupplemented fermentation. Improvements in the supplemented fermentations on a per milligram of yeasts (dry weight) basis were not due to supplementation increasing the tolerance of brewers' yeast fermentative ability to ethanol.

The enhanced death of yeasts in the high-gravity wort fermentations with increasing temperature (Fig. 3) was not an unexpected result. Ethanol and temperature effects on yeasts are known to be closely related as (i) ethanol becomes increasingly toxic to growth and viability at higher temperatures (25, 26), (ii) ethanol decreases the optimum temperature of fermentation (14), (iii) ethanol decreases the optimal and maximum temperatures for growth (36), and (iv) higher temperatures of fermentation result in decreased final ethanol concentrations in the mash (28).

The explanation for the increased inhibitory effects of ethanol at higher temperatures has been attributed to increased accumulation of intracellular ethanol at higher temperatures (26). Therefore, decreased fermentation times arising from higher temperatures of fermentation are at the price of poor yeast crop viability, which is something that would eliminate their use in brewing. In any case, the increased production of esters and higher alcohols at higher temperatures would preclude the use of temperatures above 14°C for the brewing industry (10). However, in the gasohol or distilling industries, in which end yeast crop viability is not as critical, the combination of nutritional supplementation and temperatures up to 30°C may be more practical.

Yeast crop cellular composition (especially glycogen and sterols) were found to differ considerably between anaerobic and semianaerobic conditions and to depend on the nutritional supplement used. The level of protein fell consistently within the 40 to 50% range, but the amounts of cellular glycogen and sterols fluctuated greatly. Glycogen levels were all less than 10% in yeasts under semianaerobic conditions, regardless of whether they were unsupplemented or supplemented, whereas up to nearly 30% concentrations were seen in the anaerobic fermentations (Table 1). However, the level in the fully supplemented anaerobic yeasts, which gave the fastest fermentation time (6), was 10.5%; this was the only crop under anaerobic conditions to contain less than 26% glycogen.

The level of glycogen in brewers' yeasts is an important consideration, as it is the sole source of metabolic energy for lipid synthesis and hexose transport in the first few hours of fermentation (32). Because of this, levels decline during the first 24 h of fermentation but then rise and peak at values as high as 40% at the end of the growth phase, before gradually declining during the stationary phase (19, 32). Pitching rates resulting in the presence of 160 to 200 mg of glycogen per liter are suggested to ensure enough glycogen for the synthesis of adequate lipid levels (32). Although this might suggest a potential problem for the repitching of yeasts from the supplemented fermentations, this was not found to be the case, as added lipids can compensate for low glycogen levels (Fig. 3).

Like glycogen, sterol levels varied between anaerobic and semianaerobic conditions. Levels in the semianaerobically fermented yeasts ranged from 0.85 to 1.09%, depending on the type of supplementation (Table 1), compared with levels of only 0.27 to 0.56% in the anaerobically grown yeasts. Clearly, oxygen is being introduced during the course of the semianaerobic fermentations due to the higher levels of cellular sterols in these yeasts. Brewers' yeasts normally have ca. 0.1% total sterols (dry weight) at pitching but reach 1.0% or greater within the first several hours, utilizing wort oxygen for de novo synthesis (22). The 1.0% level is a maximum ceiling which is subsequently diluted down to a 0.1% lower limit by passage of sterols to daughter cells (22).

Based on the results described above, it is clear that cellular sterol levels alone are not the growth-limiting factor in yeasts from unsupplemented high-gravity wort fermentations. In none of the yeast crops from these fermentations was the lower limit of 0.1% sterols observed. In fact, the lowest level reached was 0.48%, but this level was reached at day 6 of fermentation and remained unchanged up until the end of fermentation (day 12). It was not, therefore, the level of cellular sterols which caused the cessation in new yeast cell mass production in the unsupplemented fermentation, but other growth-limiting nutrients. In the yeasts from supplemented fermentations, the addition of the lipids in the supplement elevated even further the levels of sterols away from the growth-limiting level of 0.1%.

It remains to be determined whether the 16 to 17% (vol/vol) levels of ethanol reported here represent the true upper limit for brewers' yeasts. As sake yeast can only produce the 20 to 23% (vol/vol) levels of ethanol by the sequential addition of substrate over a period of weeks (17), it may well be possible with the sequential addition of adjunct to supplemented brewers' wort to produce beers of up to 20 to 23% (vol/vol) ethanol. Should this be possible, it would suggest the need for a complete reevaluation of the definition of alcohol tolerance in the various species of *Saccharomyces*.

In addition, it is shown that the self-imposed wort gravity limit of 16 to 18% for high-gravity brewing in industry should not be ascribed to sluggish and stuck fermentations, yeast death, or yeast intolerance to alcohol. It would appear that brewers and alcohol manufacturers could easily consider production of worts with higher gravities and enjoy larger economies of labor, capital, and energy.

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